

Screening for natural inhibitors of penicillinase by copolymerization of hydrolyzed starch or glycogen in sodium dodecylsulfate polyacrylamide gels for detecting penicillinase activity

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Abstract. The 0.08% hydrolyzed starch or glycogen were copolymerized in 7.5% or 10% sodium dodecylsulfate polyacrylamide gels. After electrophoresis and SDS removal, the commercial penicillinase in gels was reacted with penicillin G (100 mg in 50 mL, 0.1 M phosphate buffer, pH 7.0) for 30 min and then stained with 0.6% I₂ in 6% KI solutions. The clear zone of penicillinase activity bands stood out against purple or orange-red backgrounds, respectively, for hydrolyzed starch or glycogen used. This activity staining method was used successfully to detect commercial penicillinase activities from *Bacillus cereus* and the cultured methicillin-resistant *Staphylococcus aureus* ATCC 33591 strain. This activity staining method was also applied to penicillinase natural inhibitor screenings. It was found that anthraquinone-related compounds, such as aloe-emodin, emodin and rhein, could inhibit penicillinase activity. This fast and sensitive method can be used in the process of penicillinase purification, characterization and inhibitor screening.

Keywords: Activity staining; Anthraquinone; Copolymerization; Penicillinase; SDS-PAGE.

Abbreviations: SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Introduction

The β -lactam family of antibiotics includes many of the most commonly used antibacterials in clinical medicines. The majority of clinically useful β -lactams belong to either the penicillin (penam) or the cephalosporin (cepems) group (Tyczkowska et al., 1994; Lawung et al., 2001). One of the major mechanisms of resistance to β -lactams was the expression of β -lactamases which hydrolyzed the β -lactam ring. The β -lactamases (EC 3.5.2.6), such as penicillinase and cephalosporinase, which degraded penam and cepems, respectively, have been found widely in both gram-positive and gram-negative bacteria (Livermore, 1995).

Several methods were reported for β -lactamase activity determinations, such as acidimetric methods by pH stat (Labia et al., 1973), neocuproine-copper systems by spectrophotometry (Cohenford et al., 1988), nitrocefin assays by spectrophotometry (Chen et al., 1994; Lawung et al., 2001) and fluorescent spot test (Chen et al., 1994). Despite the vital role in bacterial resistance played by β -lactam antibiotics, reports concerning the activity staining of β -lactamases in gel are few. Matthew et al. (1975) used analytical isoelectric focusing for β -lactamase separations. The isoelectric focusing gel was overlaid with Whatman No.

54 paper containing chromogenic substrate, cepharosporin 87/312. After reaction, the pink β -lactamases activity bands on a yellow background were found. Tai et al. (1985) used SDS-PAGE for β -lactamase separation. The SDS-PAGE gel was overlaid with Whatman 3MM filter paper. The filter paper had been prepared by dipping in starch-iodine solution and hanging it to dry overnight. It was then stored in a dark, cool, dry place. Bonnet et al. (2000) used analytical isoelectric focusing and SDS-PAGE for β -lactamase separation. The isoelectric focusing gel was revealed with iodine-agar gel by overlaying an agar containing penicillin G (0.6%), 6% potassium iodide, and 0.6% iodine. For SDS-PAGE gels, the β -lactamases activity was detected by overlaying polyacrylamide gel containing 0.5 mM nitrocefin.

Owing to the expensive, light sensitive and water-soluble properties of synthetic substrates, such as nitrocefin, it might be suitable for detecting β -lactamases in rapid screenings. The starch-iodine filter paper for β -lactamase detection was suitable, but the preparation work was tedious. In this report, we proposed a modification of a method by Tai et al. (1985) of copolymerizing hydrolyzed starch or glycogen in SDS-PAGE gels. The penicillinase activity in gels was reacted with penicillin G and revealed by I₂-KI solutions. A number of traditional herb-derived medicines have been developed as anticancer drugs and free radical scavengers (Surh, 1999). Rhubarb, an important traditional Chinese medicinal herb, contained several anthraquinone-related compounds, such as aloe-emodin,

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emodin, and rhein. The extracts of rhubarb exhibited anti-inflammatory activities (Cuellar et al., 2001). We also used a modified penicillinase activity staining method to screen for penicillinase's natural inhibitors. We found anthraquinone-related compounds—such as aloe-emodin, emodin, and rhein—could inhibit penicillinase activity. This fast and sensitive method can be used in the process of penicillinase purification, characterization, and inhibitor screening.

Materials and Methods

Chemicals

Commercial penicillinase (type I, 5,000 units, from *Bacillus cereus*), penicillin G (sodium salt), aloe-emodin, emodin, rhein, I₂, KI, oxacillin, hydrolyzed starch (S-5651, from potato), and glycogen (type 2, from oyster) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, Bis, TEMED, APS, coomassie brilliant blue R-250, Tris, ammonium sulfate, nutrient agar (1.05450), and nutrient broth (1.05443) were obtained from E. Merck Inc. (Germany). See Blue™ pre-stained standard kit for SDS-PAGE was from Invitrogen Co. (Carlsbad, CA, USA). The kit contains myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa), and lysozyme (16 kDa). The Sephadex G-75 (F) was purchased from Pharmacia Biotech AB (Uppsala, Sweden). The strain of methicillin-resistant *Staphylococcus aureus* ATCC 33591 was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan).

Activity Staining of Penicillinase on SDS-PAGE Gels

The 0.08% hydrolyzed starch or glycogen were copolymerized in 7.5% or 10% sodium dodecylsulfate polyacrylamide gels according to Weber and Osborn (1969). The vertical mini PROTEAN 3 system (Bio-Rad Inc., USA) with a 1.00 mm thickness of spacer was used. For electrophoresis, a 75 μ l sample was mixed with 25 μ l of 60 mM Tris buffer (pH 7.0) containing 2% SDS, 14.4 mM 2-mercaptoethanol, 25% glycerol, and 0.1% bromophenol blue and then incubated at 4°C overnight. When SDS-PAGE was finished, gels were cut into two parts. One part was fixed with 12.5% trichloroacetic acid and then stained with coomassie brilliant blue R-250 dye (Neuhoff et al., 1985). The other was immersed and shaken for 10 min twice in 25% (V/V) isopropanol in 10 mM phosphate buffer (pH 7.0) to remove SDS (Hou and Lin, 1998a, 1998b; Hou et al., 1999; Hou et al., 2001; Lin et al., 2002) and finally equilibrated in 100 mM phosphate buffer (pH 7.0) for 15 min before activity staining. The method of penicillinase activity staining was as follows: The gel was soaked in the substrate solution (100 mg penicillin G in 50 mL, 100 mM phosphate buffer, pH 7.0) with gentle shaking for 30 min. After a brief rinse, the penicillinase activity was developed with 0.6% I₂ in 6% KI solutions. The clear zone of penicillinase activity bands stood out against purple or orange-red

backgrounds, respectively, for hydrolyzed starch or glycogen used. The gel was washed with 5% acetic acid for destaining.

A Methicillin-Resistant Staphylococcus aureus ATCC 33591 Cultivation

The methicillin-resistant *Staphylococcus aureus* ATCC 33591 strain was activated by nutrient broth, cultured on the nutrient agar following the manufacture's guidelines, and then stored at 4°C. A single colony was cultured in nutrient broth for 18 h, counted, and then diluted to 10⁵ cfu/mL broth. One mL of this broth was mixed with 1 mL solution containing 6 μ g oxacillin and 4% NaCl and then added to 100 mL nutrient broth by shaking at 125 rpm, 37°C for 18 h.

Partial Purifications of Penicillinase Isolated from Methicillin-Resistant Staphylococcus aureus ATCC 33591 Cultivation

The 18-h cultured broth was centrifuged at 5,000 rpm for 10 min. The pellets were recovered and then washed twice with 10 mM phosphate buffer (pH 7.0) before being resuspended in 100 mM phosphate buffer (pH 7.0) containing 0.1% Triton X-100 and then heated at 50°C for 1 h. After centrifugation at 12,000 g for 30 min, the supernatant was saved for ammonium sulfate fractionations. The precipitations of 30 to 95% ammonium sulfate were collected and then directly loaded onto a Sephadex G-75 column (1 \times 75 cm). The column was eluted with 50 mM phosphate buffer (pH 7.0). Flow rate was 30 mL/h, and each fraction contained 2 mL. Each fraction was determined for penicillinase activity using the nitrocefin method. The active fractions were pooled and concentrated with centriprep-10 to small volumes for penicillinase activity staining.

Determinations of Penicillinase Activity Isolated from Methicillin-Resistant Staphylococcus aureus ATCC 33591 Cultivation

The penicillinase activity was determined by nitrocefin methods following Lawung et al. (2001), with some modifications. Each 100 μ l of Sephadex G-75 fraction was added to 990 μ l of 100 mM phosphate buffer (pH 7.0), and then 10 μ l nitrocefin solution (10 mg in 2 mL N,N-dimethylformamide) was added with gentle mixing. The changes of absorbance at 486 nm during 3 min were recorded and expressed as $\Delta A/\text{min}$ for penicillinase activity.

Results and Discussion

A modified method of copolymerizing hydrolyzed starch or glycogen in SDS-PAGE gels was used for penicillinase activity staining and was applied to screening for penicillinase's natural inhibitors. In gauging the usefulness of this proposed method, it was considered whether the penicillinase could pass through the gel without hindrance. Figure 1 showed protein staining (A, C) and the

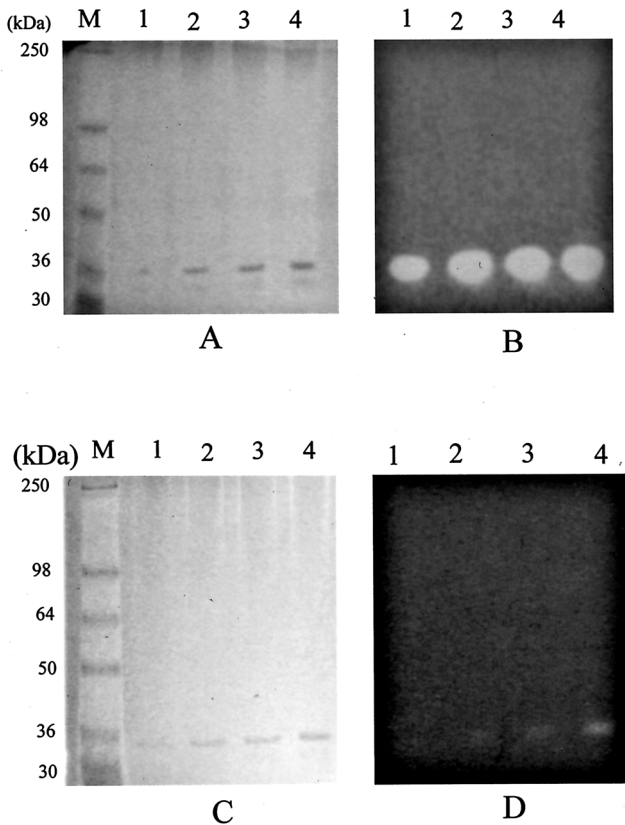


Figure 1. Protein staining (A, C) and commercial penicillinase activity staining (B, D) on 7.5% SDS-PAGE gels incorporated with 0.08% hydrolyzed starch (A, B) or glycogen (C, D). Lanes 1-4 were 0.1, 0.25, 0.5 and 0.75 units, respectively. “M” indicated the See Blue™ pre-stained markers for SDS-PAGE.

commercial penicillinase activity staining (B, D) on 7.5% SDS-PAGE gels incorporated with 0.08% hydrolyzed starch (A, B) or glycogen (C, D). Lanes 1-4 were 0.1, 0.25, 0.5 and 0.75 units, respectively. Comparing the protein staining (Figures 1A and 1C) with activity staining of penicillinase (Figures 1B and 1D), it was found that the penicillinase protein band was around 33 kDa, close to what Tai et al. (1985) reported. With increasing amounts (0.1 to 0.75 units) of penicillinase the activity bands became more intensive, meaning the incorporations of hydrolyzed starch (Figures 1A and 1B) or glycogen (Figures 1C and 1D) did not hamper the pass of penicillinase for their similar protein stainings. However, under the same conditions, the copolymerizations of hydrolyzed starch in SDS-PAGE gels had higher clear penicillinase activity than glycogen did. It might be the accessible penicillin G to penicillinase for the hydrolyzed starch used. Tai et al. (1985) used SDS-PAGE for β -lactamase separation, and then the SDS-PAGE gel was overlaid with Whatman 3MM filter paper. The filter paper had been prepared by dipping in starch-iodine solution and hanging out to dry overnight before being stored in a dark, cool, dry place. This modified method proved easier than that used by Tai et al. (1985).

This proposed activity staining method was applied to the methicillin-resistant *Staphylococcus aureus* ATCC 33591 strain for detections of penicillinase activity. After 18-h culture, the pellets were extracted with 100 mM phosphate buffer (pH 7.0) containing 0.1% Triton X-100 and then heated at 50°C for 1 h. The precipitations of 30 to 95% ammonium sulfate of bacteria lysates were collected and then directly loaded onto Sephadex G-75 column (1 × 75 cm) (Figure 2). Two minor and one major penicillinase

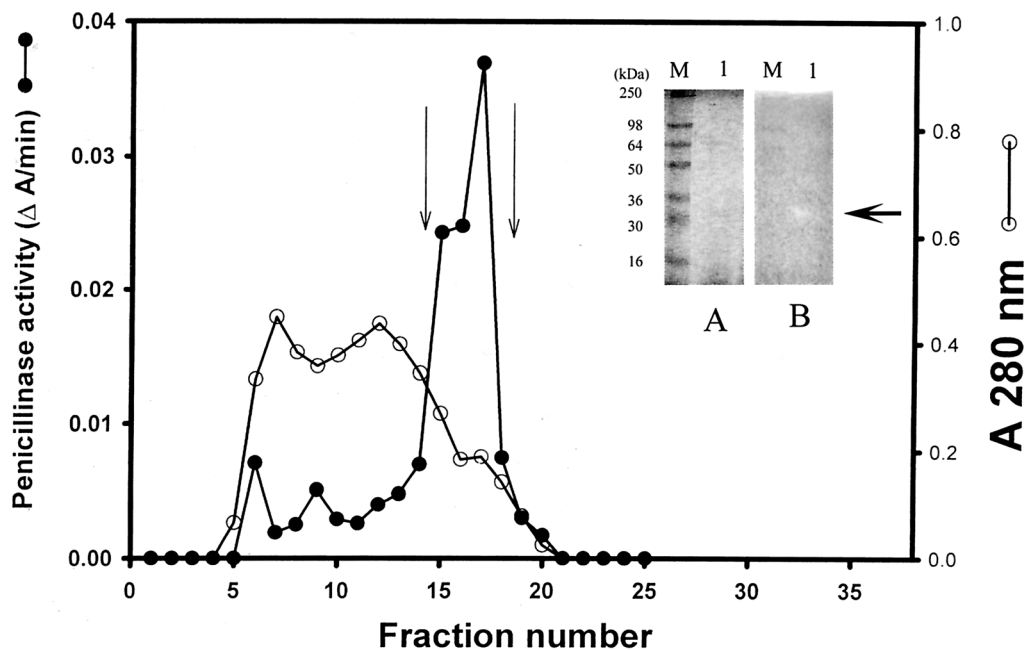


Figure 2. The chromatogram of Sephadex G-75 column (1 × 75 cm) on penicillinase activity ($\Delta A/min$) of the methicillin-resistant *Staphylococcus aureus* ATCC 33591 strain of 30 to 95% ammonium sulfate by nitrocefin methods. The inset showed protein staining (A) and penicillinase activity staining (B) on 10% SDS-PAGE gels incorporated with 0.08% hydrolyzed starch. “M” indicated the See Blue™ pre-stained markers for SDS-PAGE. Each well contained 24 μg proteins. The arrow indicated the position of penicillinase activity band.

activity bands were found using nitrocefin methods. These major penicillinase activity fractions were pooled, concentrated and then applied for activity staining on 10% SDS-PAGE gels incorporated with 0.08% hydrolyzed starch (Figure 2, inset). It was found that the clear zone of the penicillinase activity band (Figure 2, inset B, arrow indicated) was around 33 kDa, similar to that Tai et al. (1985) reported. One of the major mechanisms of resistance to β -lactams was the expression of β -lactamases, which hydrolyzed the β -lactam ring. It was clear that the methicillin-resistant *Staphylococcus aureus* ATCC 33591 strain expressed penicillinase activity against 6 μ g oxacillin in the cultured medium.

Rhubarb, an important traditional Chinese medicinal herb, contained several anthraquinone-related compounds such as aloe-emodin, emodin, and rhein. The extracts of rhubarb exhibited anti-inflammatory activities (Cuellar et al., 2001). We also used the penicillinase activity staining method detailed here to screen for natural inhibitors (Figure 3). Lane 1, commercial penicillinase (0.75 units) as the controls; lanes 2 to 4 were aloe-emodin, emodin and rhein, respectively, added to commercial penicillinase for 12 h. Compared with protein staining (Figures 3A and 3C),

it was found that anthraquinone-related compounds, aloe-emodin, emodin, and rhein (1 mg/mL, lanes 2 to 4), could inhibit penicillinase activity, especially rhein (Figures 3B and 3D). Based on the results of activity staining of commercial penicillinase from *Bacillus cereus* (Figure 1) and from methicillin-resistant *Staphylococcus aureus* ATCC 33591 strain (Figure 2), the modified penicillinase activity staining method, the copolymerizing of hydrolyzed starch or glycogen in SDS-PAGE gels, presented here seems more convenient than that reported by Tai et al. (1985). We also applied this method to penicillinase inhibitor screenings. This fast and sensitive method can be used in the process of penicillinase purification, characterization, and inhibitor screening.

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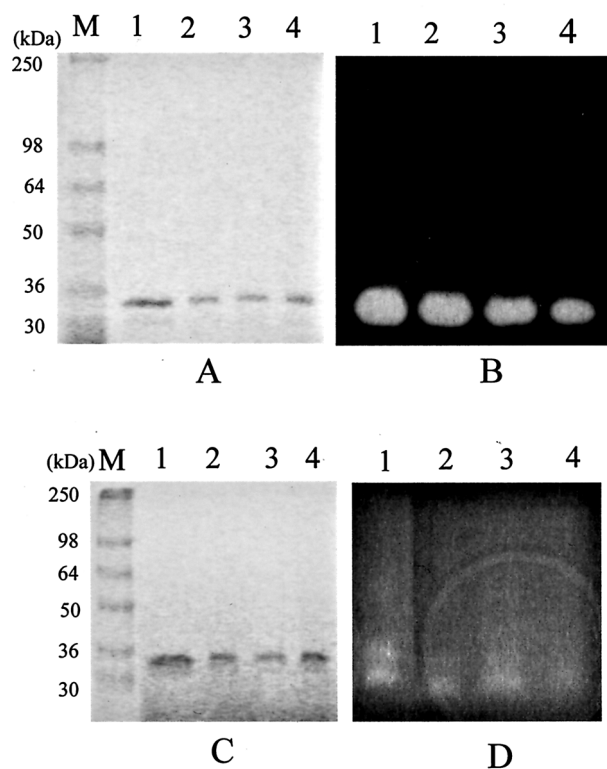


Figure 3. Protein staining (A, C) and commercial penicillinase activity staining (B, D) on 10% SDS-PAGE gels incorporated with 0.08% hydrolyzed starch (A, B) or glycogen (C, D). Lane 1, commercial penicillinase (0.75 units) as the controls; lanes 2 to 4 were aloe-emodin, emodin and rhein (1 mg/mL in dimethylformamide), respectively, added to commercial penicillinase for 12 h. "M" indicated the See Blue™ pre-stained markers for SDS-PAGE.

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以水解過之澱粉或肝醣共聚合於 SDS-聚丙烯醯胺膠體中檢測盤尼西林水解酶活性作為盤尼西林水解酶天然抑制物之篩選

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以 0.08% 水解過澱粉或肝醣共聚合於 7.5% 或 10% SDS-聚丙烯醯胺膠體中。經過電泳與移除 SDS 的步驟後，在膠體中的盤尼西林水解酶和盤尼西林 G（100 mg 在 50 mL，0.1 M 磷酸緩衝液，pH 7.0）反應 30 分鐘後，以 0.6% I₂ 在 6% KI 溶液中染色。盤尼西林水解酶分別在紫色（水解過澱粉）或是橘紅色（肝醣）的背景色下，呈現反白的活性帶。利用此活性染色法，檢測商品化盤尼西林水解酶（來自 *Bacillus cereus*）以及自行培養的 methicillin-抗性的 *Staphylococcus aureus* ATCC 33591 株。利用此活性染色法運用於盤尼西林水解酶天然抑制物之篩選。結果顯示，發現蒽醌類（anthraquinone-related）化合物，例如 aloe-emodin, emodin and rhein，都能抑制盤尼西林水解酶的活性。此一快速且靈敏的方法可以運用於盤尼西林水解酶的純化、特性分析及天然抑制物的篩選上。

關鍵詞：活性染色；共聚合；盤尼西林水解酶；蒽醌類。